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The Effect of Ultrasound on Botulin Toxin

by

G. Scheibner

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MUL 0567

The Effect of Ultrasound on Butulin Toxin

by

Dr. G. Scheibner, Leipzig

Bacteria and viruses have frequently been the subject of studies on the effects of ultrasound. A wide variation of frequencies and various intensities of sound were used in these works. So far as I am aware, among bacterial toxins so far only the tetanus and diphtheria toxins have been exposed to ultrasound.

In 1938, Kasahara and Takagi (3) reported the weakening of the diphtheria toxin by ultrasound waves. Also from Japan, Kasahara, Sha-Shi-Nan and Kakusua (2) studied the effect of ultrasound waves on the tetanus toxin. At a frequency of 450 KHz and a current of 2100 V, complete detoxification of the toxin could be achieved by 3 min. ultrasound exposure of dry toxin (0.001 g) dissolved in physiologic saline. Gligorijevic and Tvoric (1), on the other hand, were able to reduce the toxicity of tetanus toxin for laboratory animals with frequencies of 800-3,000 KHz and intensities of 1.5-3 W/cm², but were unable to achieve complete inactivation.

The object of the present work was to pursue the works cited above by investigating the effect of ultrasound

waves on another bacterial toxin, namely botulin. In case of the possible killing of the toxin, we further wished to investigate whether the antigenic properties persisted and whether the toxin exposed to ultrasound could thus be used for purposes of immunization as a toxoid.

Materials and Methods

Type A and C toxins of *Cl. botulinus* were used for exposure to ultrasound. The type C toxin was a crude toxin in the form of a supernatant from liver-liver bouillon inoculated with Type C Clostridia and incubated for 6 days at 37°C, obtained after vigorous centrifugation (30 min at 3,000 rpm). Type A toxin, following the method of Sterne and Wentzel (see 5) was cultured in a dialysis tube, in other words it was a purer toxin than type C. This toxin was also vigorously centrifuged and the liquid supernatant was used for ultrasound. Both toxins were evaluated on albino mice before exposure to ultrasound. With type A a minimum lethal dose of ca. $0.5 \cdot 10^{-1}$ cc could be achieved after intra-abdominal injection, while with type C, $0.5 \cdot 10^{-3}$ cc injected i.m. to albino mice was still lethal. When injected s.c. to albino mice, both toxins showed less effect. The weakening was roughly by a power of ten. The toxins exposed to ultrasound were also injected s.c. to experimental mice. Thus ultrasound

could only be applied in the case of type A up to a dilution of 10^{-3} and in the case of type C up to 10^{-2} , so as to have exact controls with toxins not exposed to ultrasound.

The source of ultrasound was a 1.5 KHz ultrasound industrial generator (Type 602, No. 1030) manufactured by R-F-T-Funkwerk, Erfurt, kindly made available to us by the Physics Institute of the Karl Marx University of Leipzig*. The generator was furnished with a sound vessel from the Zeiss Company, Jena, and worked at a frequency of 800 KHz. The ultrasound vessel used was a sound beaker from the Zeiss Company, Jena, with a $\lambda/2$ -bottom and an aperture of 41 mm. Particular stress had to be placed on adequate cooling of the toxin material during ultrasound application, so as to prevent weakening, or indeed killing of the toxin by a pure heat effect. In preliminary experiments, therefore, we measured the temperature in non-inoculated liver bouillon for various initial voltages and varying periods of sound exposure. It was found that at the maximum intensity of ca. 2.42 W/cm^2 achievable with the generator after ultrasound application of 20 min duration, the liver bouillon exposed to ultrasound had a temperature of 41°C . In initial experiments with toxin or diluted toxin, however, the temperature increased in some cases to considerably

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higher values (up to 68°C). Thus in addition to the water-cooling normally used in the sound vessel, added ice cooling was used, in such a way that the sound beaker referred to above, containing the liquid to be exposed to ultrasound, was placed in a large sound beaker also furnished with a $\lambda/2$ -bottom and the surrounding space was filled with ice fragments, which could be supplemented as needed. The temperature of the ultrasound liquid was measured during and at the end of ultrasound exposure. As a result of the strong cooling, in some cases lower final temperatures were measured than before ultrasound exposure. Thus adequate cooling was provided. Quantities of liquid from crude toxin and from the toxin dilutions of 25.0 cc were used for ultrasound exposure. Evaluation of the toxins exposed to ultrasound was made on albino mice by inoculation of 0.5 cc of the material in question to 2 mice each.

Experiments

We first worked with type C toxin. Additional ice cooling was not used in these experiments. The intensity of sound, period of sound exposure, final temperature and outcome of the experiments are shown in Table 1.

Table 1: Ultrasound experiments with type C botulin toxin
without ice cooling

1. Toxinverdünnung	2. Intensität	3. Behandlungszeit	4. Endtemperatur d. beschallten Flüssigkeit	5. Ausgang des Tierversuches	
6. Rohtoxin	0.5W/cm ²	5 Min.	30° C	†	†
"	0.5W/cm ²	20 "	31° C	†	†
"	2.4W/cm ²	5 "	43° C	†	†
"	2.42W/cm ²	20 "	46° C	†	†
"	7 unbeschalt. zur Kontrolle			†	†
10 ⁻¹	0.5W/cm ²	5 Min.	31.5° C	†	†
10 ⁻¹	0.5W/cm ²	20 "	34° C	†	†
10 ⁻¹	2.42W/cm ²	5 "	43° C	†	†
10 ⁻¹	2.42W/cm ²	20 "	63° C	†	†
10 ⁻¹	7 unbeschalt. zur Kontrolle			†	†
10 ⁻²	0.5W/cm ²	5 Min.	28.1° C	†	†
10 ⁻²	0.5W/cm ²	20 "	33° C	†	†
10 ⁻²	2.42W/cm ²	5 "	41° C	†	†
10 ⁻²	2.42W/cm ²	20 "	65.5° C	0	0
10 ⁻²	7 unbeschalt. zur Kontrolle			†	†

8 Zeichenerklärung:

9 z. B. † Maus gestorben innerhalb z. B. 12 Std. p. i.
10 0 Tier blieb gesund

Key:

1. Toxin dilution
2. Intensity
3. Period of sound exposure
4. Final temperature of ultrasound fluid
5. Outcome of animal experiments
6. Crude toxin
7. Control without ultrasound exposure
8. Explanation of symbol
9. e.g. †12 = mice died for instance 12 hours after injection
10. 0 = animal stayed healthy

Since at an initial voltage of 2.2 KV after ultrasound exposure of 20 min duration the toxin was killed (dilution stage 10⁻³) or weakened (dilution stage 10⁻¹), the object was to investigate whether this was merely a heat effect, or whether the change in the toxin was caused by the influence of the ultrasound waves. Therefore additional ultra-

sound waves. Therefore additional ultrasound applications were made, but this time with the added ice cooling referred to above. These experiments are shown in Table 2.

Table 2. Ultrasound experiments with type C botulin toxin, with ice cooling.

Key: See Table 1.

Toxinver- dünnung	Intensität	Beschal- lungszeit	Endtempera- tur d. beschal- ten Flüssigkeit	Ausgang des Tierversuches	
10 ⁻¹	2,42W/cm ²	20 Min.	29° C	+ 18	+ 13
10 ⁻¹	2,42W/cm ²	60 Min.	44° C	+ 18	+ 18
10 ⁻¹	unbeschallt als Kontrolle		-	+ 18	+ 18
10 ⁻²	2,42W/cm ²	60 Min.	32° C	+ 36	+ 36
10 ⁻²	unbeschallt als Kontrolle		-	+ 36	+ 36

Zeichenerklärung siehe Tabelle 1

It can be seen from the experiments that the weakening of the toxin observed after prior ultrasound exposure can be attributed solely to the heat effect.

The botulin A toxin obtained in a dialysis tube was used for ultrasound experiments in dilutions of 10⁻² and 10⁻³, since according to the results with ultrasound application to toxin C, it was anticipated that the effect of ultrasound waves on undiluted A toxin would not be detectable in animal experiments. Also based on our experiences with the results of ultrasound on C toxin, we worked only at the highest intensity and with prolonged exposure to ultrasound. Table 3 shows the ultrasound experiments with A toxin.

Table 3. Ultrasound experiments with type A botulin toxin with ice cooling

Key: See Table 1

Toxinver- dünnung	Intensität	Beschal- lungszeit	Endtempera- tur (beschal- lungsfähigkeit)	Ausgang des Versuches	
10 ⁻¹	2.42 W/cm ²	60 Min.	33° C	13	13
10 ⁻²	unbeschallte Kontrolle	-	-	13	18
10 ⁻³	2.42 W/cm ²	60 Min.	30° C	0	0
10 ⁻³	unbeschallte Kontrolle	-	-	24	36

Thus A toxin could be weakened by roughly a power of 10 by one hour of exposure of ultrasound.

In the experiments so far, always 25.0 cc toxin or toxin dilution was exposed to ultrasound. But we were also interested in the effect of ultrasound waves on quantities of smaller volume. Quantities of 5.0 cc were exposed to ultrasound in an ordinary test tube (opening 14 mm, glass thickness 0.5 mm). The permeability of the glass to ultrasound waves was established in preliminary experiments and was roughly the same as that of the sound beaker with the $\lambda/2$ -bottom. Both with type A and type C, toxin dilutions of 10^{-2} were chosen. This dilution stage was still just lethal for albino mice in the case of type C and was also capable of killing the mice with type A after one hour's ultrasound exposure of 25.0 cc toxin dilution at an intensity of 2.42 C/cm^2 , while with type A the mice injected with the ultrasound-exposed toxin dilution of 10^{-3}

did not die (see Table 3).

This experiment was also carried out with ice cooling. The experimental procedure and results are shown in Table 4.

Table 4. Ultrasound experiments with small volumes of toxin dilutions of type A and C botulin toxins, with ice cooling.

1	2	3	4	5	6
Toxinverdünnung	Toxintyp	Intensität	Beschallungszeit	Endtemperatur des beschallten Flüssigkeit	Ausgang des Tierversuchs
10:2	A	2.42 W/cm ²	60 min.	6.9 °C	24
10:4	A	7 unbeschallte Kontrolle	-	-	24
10:2	C	2.42 W/cm ²	60 min.	6.9 °C	18
10:2	C	unbeschallte Kontrolle	-	-	30

8 Zeichenerklärung siehe Tabelle 1

- Key:
1. Toxin dilution.
 2. Type of toxin
 3. Intensity
 4. Period of ultrasound exposure
 5. Final temperature of the ultrasound liquid
 6. Outcome of animal experiment
 7. Control without ultrasound exposure
 8. For explanation of symbols see Table 1.

Discussion

The outcome of the experiments shows that at the maximum intensity of ca. 2.42 W/cm² achievable with the ultra-

sound generator used, no weakening of the type C botulin obtained in liver bouillon could be achieved. On the other hand the type A toxin obtained in a dialysis tube, at the same intensity and with a duration of ultrasound exposure of 1 hour, led to a weakening by about a power of 10. The method of obtaining toxin in a dialysis tube, devised by Sterne and Wentzel (see 5), is based on the fact that a dialysis tube coated with physiologic saline is immersed in liver bouillon as surrounding culture medium. The dialysis tube and liver bouillon are in a large culture vessel. The saline solution in the tube is inoculated. Therefore from the surrounding liver bouillon only the substances in genuine solution can diffuse through the tube and serve as culture media for the Clostridia. Thus the toxin obtained in the dialysis tube is a purer toxin, since in this case the protein contained in ordinary liver bouillon is not contained in the crude toxin. The differences in the results of ultrasound exposure in toxin type A and C are less likely to be explained by the supposed difference in the toxin molecules (the type A molecule was recognized as globulin with a molecular weight of ca. 900,000), but by the difference in media used to obtain the toxins. The proteins contained in ordinary liver bouillon may work on the toxin molecule in the manner of a haptogenic membrane, and thus in the manner of a protective covering against ultrasound waves. In addition, the large amount of reducing substance

contained in liver bouillon might at least partially eliminate the oxidizing effect of ultrasound waves and thus largely prevent any oxidizing effect on the toxin molecule.

In any event it can be seen from the results of our experiments that one hour of ultrasound exposure at an intensity of ca. 2.42 W/cm^2 is not sufficient to bring about deactivation of the botulin toxin. However, it may be concluded from the weakening of the type A toxin that with a more prolonged exposure to ultrasound and above all with a higher intensity, it should definitely be possible to achieve an even more marked weakening of the toxin, if not a complete deactivation. Unfortunately the maximum attainable intensity of the ultrasound industrial generator used was only about 2.42 W/cm^2 , so that we were unable to use greater intensities.

Summary

The toxicity of type A botulin toxin obtained in a dialysis tube could be reduced by roughly a power of 10 by one hour of exposure to ultrasound waves (frequency 800 KHz, intensity ca. 2.42 W/cm^2). Type C toxin obtained from liver bouillon showed no reduction of toxicity for albino mice after the same ultrasound exposure. The difference in behavior may be due to the differences in the media used.

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